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Prognostic Factors for Fibromatoses: A Correlation of Proliferation Index, Estrogen Receptor, p53, Retinoblastoma, and *src* Gene Products and Clinical Features With Outcome

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Background: The aggressiveness of fibromatoses is difficult to predict by morphologic analysis. Additional prognostic markers would be helpful for clinical management.
Materials and Methods: Proliferation index (MIB-1), p53, *src*, retinoblastoma gene protein products, estrogen receptor level, site and depth of lesion were correlated with incidence of recurrence in 52 patients. Superficial (47) and deep (5) fibromatoses were studied. Anatomic sites included the extremities, head, neck, trunk, and pelvis.
Results: Twenty (38%) lesions recurred locally. All five deep lesions recurred, but only 32% of superficial tumors recurred. Mean proliferation index for recurrent lesions was 0.82% and 0.73% for nonrecurrent fibromatoses; no significant differences were observed. Five recurrent lesions (25%) expressed estrogen receptor >5 fmol/mg as did 31% (10 of 32) of the nonrecurrent lesions. None of the tested specimens expressed *src* gene product. Eight of the lesions which recurred (40%) contained p53, but only five nonrecurring tumors (16%) expressed p53. One of five deep lesions (20%) expressed p53 and 26% (12 of 47) of superficial tumors expressed p53. Forty-six percent (6 of 13) of recurrent lesions tested were retinoblastoma protein product negative, but only 33.3% (7 of 21) of nonrecurring tumors were retinoblastoma protein product negative.
Conclusions: Only p53 and depth of lesion were of statistical value for the prediction of recurrence. *J. Surg. Oncol. 1997;65:117–122.*

KEY WORDS: **fibromatosis; proliferation index; p53; ER; prognostic factors**

INTRODUCTION

Fibromatoses are benign proliferations of fibroblasts and myofibroblasts which are characterized by an infiltrative growth pattern but a bland cytologic appearance and low mitotic rate [1]. The majority of these proliferations can be successfully treated by conservative local excision, but some are capable of recurrence. Deep lesions are more likely to recur than superficial ones but there appear to be no reliable morphologic findings useful for the prediction of recurrence [1]. Other prognostic markers including proliferation index (MIB-1), PCNA score, p53, and DNA ploidy have met with limited success in estimating the likelihood of recurrence for fibromatoses [2].

We investigated the prognostic value of p53, *src* protein product, retinoblastoma (Rb) protein product, estrogen receptor (ER) level, and proliferation index as determined by MIB-1 in a series of 52 cases of fibromatosis.

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Five were deep-seated lesions while 47 were categorized as superficial. Twenty of the 52 (38%) recurred locally. Correlation was made between the presence of these prognostic markers and clinical outcome.

MATERIALS AND METHODS

The Duke University Medical Center Pathology Department files were searched for all cases of fibromatosis resected between 1989 and 1992. Fifty-two cases were found and the paraffin blocks obtained. Follow-up of at least 3 years duration was available for all patients.

Proliferation Index

Proliferation index was determined with the monoclonal antibody MIB-1 (AMAC, Inc., Westbrook, ME) diluted 1:100 (1 μg/ml in phosphate-buffered saline, pH 7.4 [PBS; Sigma Chemical Co., St. Louis, MO] with 2% bovine serum albumin [2% BSA/PBS]). Dilutions for the primary antibody were based on titration studies utilizing reactive lymph nodes known to have a high proliferation index in the germinal centers. A negative control was run by diluting a nonimmune specific IgG_1 mouse isotype antibody (Coulter Source, Inc., Marietta, GA) 1:100 (1 μg/ml in 2% BSA/PBS). Histologic material from all cases was examined by a single pathologist (L.J.L.) for sample adequacy. Serial sections cut 5 μm thick were mounted on +Plus + slides (Fisher, Charlotte, NC). The slides were dried overnight at 60°C in an Imperial Oven (Baxter, McGaw Park, IL). Specimens were deparaffinized in three successive xylene baths and cleared in absolute ethanol. The slides were brought to water and immersed in a microwaveable pressure cooker with 1,500 ml of 10 mM citrate buffer (pH 6.0). The unit was heated in a 700 W microwave (Quasar model MQ7677BW, Elk Grove, IL) for 30 minutes at the highest power setting. This was followed by a 30-minute cooling period. Slides were slowly brought to water, trying to avoid any drastic temperature change.

The assay slides were rinsed with PBS and then placed in 5% normal goat serum (NGtS) for 20 minutes. Excess NGtS was blotted from the slides and the primary antibodies (IgG_1 MIB-1) were applied and incubated overnight at 4°C for approximately 18 hours. All of the assay incubations were carried out in a humidity chamber to prevent solution evaporation. After bringing the slides to room temperature, they were rinsed in PBS three times for 5 minutes each rinse. Subsequently, goat anti-mouse biotinylated antibody (BioGenex, San Ramon, CA) was applied for 35 minutes. The slides were again rinsed in PBS three times for 5 minutes each rinse, followed by application of peroxidase-conjugated streptavidin label (BioGenex) for 35 minutes. Next, they were rinsed in PBS three times for 5 minutes each rinse. The slides were then developed for 5 minutes with the chromogen 3,3′ diaminobenzidine (Sigma; 0.5% diaminobenzidine [DAB] in 0.05 M Tris buffer and 0.6% hydrogen peroxide). Finally, the assay slides were rinsed in running tap water for 10 minutes, counterstained with 1% methyl green in 0.1 N sodium acetate buffer, pH 5.2 (Sigma), dehydrated in butanol, and coverslipped in Pro-Texx™ (Baxter).

Determination of Proliferation Index

The MIB-1 proliferation index was determined using a CAS 200D™ Image analysis System (Becton Dickinson-Cell Analysis Systems [BD-CIS], San Jose, CA) in combination with the quantitative Proliferation Index Software Program (BD-CIS). Quantitation of MIB-1 immunoreactivity has been previously described for formalin-fixed, paraffin-embedded tissue sections [3]. Briefly, the data were obtained by computerized image analysis of the MIB-1 immunostained slides and were expressed as proliferation index (PI), where PI is the percent of nuclear area positively stained with the MIB-1 monoclonal antibody relative to the total nuclear area. Foci of tumor were identified and two measurements were taken, one with 10 consecutive fields and the other with 15 consecutive fields. The fields were selected on the basis of tumor viability and the analysis was started in an area where there was obvious proliferation. The fields were analyzed at a magnification of 400×. The automated mean of MIB-1 served as the global tumor score and was representative of the proliferation index for the tumor. A small amount of heterogeneity in the staining intensity was noted within some tumor samples and was most likely due to an artifact of fixation or microtomy. As a result of sectioning, the amount of cell nucleus present was variable. The amount of cell nucleus present determined how intense the cell was stained, thus producing stain intensity heterogeneity. Control sections stained with nonspecific mouse isotype IgG_1 were prepared in each case. The controls were evaluated by standard light microscopy and analyzed by image analysis to establish background immunostaining thresholds for the areas analyzed.

ER

ER levels were obtained by quantitative immunohistochemistry on formalin-fixed paraffin-embedded tissue. Five micron sections were cut and placed on ''+Plus+'' slides (Baxter). The slides were oven dried overnight at 60°C and then cooled for 30 minutes. They were deparaffinized in three cycles of xylene and then rehydrated in three changes of ethanol. The slides were brought to water and were immersed in a microwaveable pressure cooker with 1,500 ml of 10 mM citrate buffer (pH 6.0). The lid of the pressure cooker was tightly sealed and the unit was heated in a 700 W microwave (Quasar) set on high for 30 minutes. It is critical that during the last 10–12 minutes of the 30-minute microwaving process

that boiling occurs, this can be confirmed through the movement of the rubberized weight of the pressure cooker as it releases steam. Upon completion of the microwaving process, the rubberized weight is removed to allow the complete release of steam. This is followed by a 30-minute cooling period (15 minutes with the lid on and 15 minutes with the lid off) (patent no. 5,244,787).

Automated ER Immunohistochemistry Assay

Upon completion of the cooling process, slides were washed in two changes of Ventana wash buffer solution (Ventana Instruments Co., Tucson AZ) and the appropriate bar code label was applied to each slide. The slides are then attached horizontally by metal clips on a level carousel within the temperature-equilibrated reaction chamber. The Ventana ES 320™ is activated by loading the preprogrammed ER/PgR IHC (progesterone receptor immunohistochemistry) recipe file. Each recipe file consists of a specific sequence of buffer rinses, enzyme inhibitors, blocking serums, antibodies, detection complexes, chromogens, and counterstains which were used according to the manufacturer's instructions.

After the initial series of buffer rinses and normal serum preincubations, the ER primary antibody was dispensed using a 100 μl dose per slide volume mechanical plunger. The specific antibody was localized by a universal anti-mouse secondary IgG-biotinylated antibody cocktail (Ventana). This step was followed by a streptavidin-enzyme conjugate and visualized as a brown stain with DAB chromogen with a copper sulfate enhancement. Each step was incubated for a precise amount of time and at a 42°C standardized temperature. At the end of each incubation step, the instrument rinsed the sections to stop the reaction and remove unbound material that could potentially cause background. Following the automated staining process, the slides were rinsed in tap water followed by sodium acetate incubation and then counterstained with 1.0% methyl green in 0.1 sodium acetate buffer, pH 5.2 (Sigma), for 5 minutes. The nuclear counterstain methyl green was chosen since it provides the best spectral separation from the brown DAB chromogen. The slides were then coverslipped and labeled.

Image ER Quantitation

Quantitation was achieved by measurement with a CAS 200D™ computerized image analyzer, using the quantitative ER/PgR Beta-Software Package Version 2.51 (BD-CIS). The instrument and its operation have been previously described [4,5]. A light microscope is linked to an interactive computer and measurements are acquired through the use of specific software applications. Two cameras with two bandpass filters, one at 620 nm which measures all nuclei stained with methyl green (with or without DAB staining) and the other at 500 nm which measures only nuclei stained with DAB, allow for excellent spectral discrimination between the brown (DAB chromogen) and green (methyl green). The ER/PgR software application is used to measure the percentage of cell nuclei in the tissue section having ER, and the density or concentration of receptor in those nuclei. A determination of heterogeneity of staining from field to field is also provided.

The image measurement takes into account not only the amount of nuclear area covered in DAB chromogen, but also how intense the chromogen is staining by determining the percentage of light transmission (%T) through the nuclear area. The less light transmitted through the nuclear area, the more DAB chromogen or staining is present in that nuclear area, thus measuring the level of receptor present.

Both nuclear and antibody thresholds were set with the negative control antibody cocktail slide. The nuclear threshold was set to the value that best discriminated between the nuclei and cytoplasm. The antibody threshold was set to the value at which no stain could be detected in the nuclei of the negative control slide.

Standardization between immunohistochemical assays for the fmol/mg values was established through the use of a calibrator and two control tumor tissues in each assay. Additionally, the calibrator and control tissues were treated in the same manner as the sample tissue. This calibration and control tissue originated from tumor blocks with adequate tumor volume and distribution and a known ER value as predetermined by the dextran coated charcoal (DCC) ligand-binding assay. A one-point linear calibration was utilized during the assay of sample tissue. Quantitation was performed on 10 fields which contained DAB staining for each sample tissue.

p53, Rb, and *src*

The material used for the p53, Rb, and *src* portions of the study was the same set of paraffin blocks as used for the MIB-1 determinations. The antibodies used included the p53 clone PAb1801 (AB-2; Oncogene Science, Manhasset, NY), which is an affinity-purified IgG_1 monoclonal antibody that recognizes a denaturation-resistant epitope in the human p53 protein located between amino acids 32 and 79 [6]. This antibody was used at 1.0 μg/ml on paraffin-embedded material and at 0.5 μg/ml on fresh tissue. In each, serial sections were reacted with mouse IgG_1 (1.0 μg/ml for paraffin assays and 0.5 μg/ml for fresh tissue assays) to assess nonspecific staining.

Slides were heated to 65°C in an Imperial III Incubator (Lab-Line, Melrose Park, IL) for 60 minutes. The slides were then taken directly from the incubator and deparaffinized in three changes of xylene for 5 minutes each, followed by three rinses absolute ethanol for 2 minutes each. To eliminate staining due to endogenous peroxi-

TABLE I. Correlation of MIB-1 Proliferation Index With Recurrence of Fibromatosis

Proliferation index (%)	Total cases	No. of cases recurring	No. of cases not recurring
0.1 to 0.5	37	15	22
0.6 to 1.0	6	2	4
1.0 to 5.0	2	1	1
>5.0	2	1	1

dase, sections were treated with an ethanol/H_2O_2 (45 ml ethanol/3 ml H_2O_2) block for 10 minutes at 42°C. Slides were hydrated in decreasing concentrations of ethanol and rinsed in PBS. From this point in the assay, all antibody and complex incubations were carried out in a humidity chamber at 42°C. Slides were preincubated for 10 minutes in 5% normal human serum (NHS) diluted in 2% BSA/PBS followed by an incubation for 45 minutes with the primary antibody. Slides were then rinsed in PBS and subsequently incubated in the presence of the secondary antibody for 20 minutes. The secondary antibody was a biotinylated, affinity-purified horse anti-mouse IgG (Vector Labs, Burlingame, CA) used at a dilution of 1:100 in 2% BSA/PBS. Slides were rinsed in PBS followed by a 20-minute incubation in premixed Elite Universal Kit reagents as recommended by the product insert (Vector Labs). The PBS rinse was repeated and sections were developed in the enzyme substrate DAB solution. After chromogen development, slides were washed in running tap water for 10 minutes and counterstained with 1% methyl green (Sigma) in sodium acetate buffer, pH 5.2. Slides were then dehydrated, cleaned in xylene, and mounted in permanent coverslipping media (Pro-Texx, Baxter). Slides were graded as positive or negative by a single pathologist (L.J.L.).

The *src* oncogene is a product of the avian sarcoma virus (pp 60 *src*) which possesses characteristic protein kinase activity with specificity for tyrosine residues. The antibody to *src* is a purified Igb (Oncogene Science, Uniondale, NY). The antibody (titer of 1:100) was prepared and run in a fashion similar to that described for MIB-1. The antibody directed against the Rb protein product was obtained from Oncogene Science and run as described above, but using a titer of 1:50.

RESULTS

In all cases, positive nuclear staining for MIB-1 was recognized within the nuclei of variable percentages of the neoplastic cells. The distribution of the positivity within the cell populations appeared to be random. Table I correlates quantitated MIB-1 values with recurrence. There was no relationship between MIB-1 level and recurrence, and no level of MIB-1 immunoreactivity could be found which predicted recurrence. The majority of cases demonstrated a proliferation index (MIB-1) of 0.1–0.4%. The mean PI for recurrent lesions was 0.82% and for nonrecurrent neoplasms 0.73%. Even at cutpoints set near the highest PIs (5.0%), there was no prognostic distinction between recurrent and nonrecurrent fibromatoses.

Immunohistochemical assays for the *src* gene product were performed on 20 lesions (8 recurrent, 12 nonrecurrent) and all were negative for this protein product. Immunohistochemical determinations for the Rb gene protein product were performed in 34 patients (13 recurrent and 21 nonrecurrent lesions). Twenty-one neoplasms expressed the Rb protein. Forty-six percent of lesions which recurred (6 of 13) failed to express the Rb protein and 33.3% of nonrecurring fibromatoses failed to express this protein product. This difference did not reach statistical significance ($P = 0.49$). Two of the three deeply situated (66.6%) and 11 of 29 (35.5%) superficial lesions tested were negative for the Rb protein product. When only superficially located fibromatoses were considered, 60% of recurring lesions expressed the Rb protein product and only 27% (3 of 11) of Rb-negative lesions recurred. Retinoblastoma gene product status did not appear to predict recurrence for fibromatoses within the entire set or within the subcategories of superficially or deeply located lesions ($P = 1.0$).

Five of the recurrent lesions (25%) expressed ER levels higher than 5 fmol/mg and 31% of nonrecurrent fibromatoses expressed ER levels above 5 fmol/mg (Table II). Twenty-eight percent (13 of 47) of superficial lesions had ER levels higher than 5 fmol/mg and two of five (40%) deep lesions demonstrated ER levels above 5 fmol/mg. No statistically significant relationships existed between ER level and the recurrence or depth of fibromatoses ($P = 0.8$; Table III).

Thirteen cases (25%) contained recognizable accumulations of p53. Eight of the recurring lesions (40%) contained p53 while only five nonrecurring lesions (16%) contained measurable amounts of p53 (Table II). Twenty percent of deeply located fibromatoses (1 of 5) expressed measurable p53 and 26% of superficial lesions contained demonstrable p53. The difference in p53 expression by recurring and nonrecurring fibromatoses was nearly statistically significant ($P = 0.06$).

All five deep lesions recurred, but only 32% (15 of 47 cases) of superficially located lesions recurred. This represented a statistically significant difference ($P = 0.006$).

DISCUSSION

Fibromatoses are low-grade proliferations composed of fibroblasts, myofibroblasts, and collagen fibers. They are separated from low-grade fibrosarcomas by differences in cellularity, mitotic rate, and some differences in architectural pattern [1]. While fibromatoses do not display metastatic behavior, they may demonstrate multiple

TABLE II. Correlation of Prognostic and Clinical Markers With Recurrence in 52 Cases of Fibromatosis

	MIB-1	ER (>5 fmol/mg)	Deep location	C-*src* +	Rb +	p53 +
Recurrent	5 (25)[a]	5 (25)	5 (25)	0 (0)	7 (35)	8 (40)
Nonrecurrent	11 (34)	10 (31)	0 (0)	0 (0)	14 (44)	5 (16)
Total positive	16	15	5	0	21	13
Total cases tested	52	52	52	20	34	52

[a]Percent of cases positive for prognostic marker.

TABLE III. Prognostic Significance of Tested Markers for the Prediction of Recurrence of Fibromatosis

Marker	*P* value
MIB-1	0.22
ER	0.80
p53	.06
Depth	.006
RB	.49
src	1.0

local recurrences with associated morbidity and mortality. While location has some predictive value in estimating chances of recurrence, morphologic analysis is of little or no help in separating lesions which are likely to recur from those which are not. A variety of markers have been discovered which are predictive of aggressiveness for a spectrum of neoplasms. Among these markers, proliferation index (MIB-1), p53, Rb gene product, *src* gene product, and ER can be assayed in paraffin-embedded tissue by immunohistochemical techniques and quantitated in an objective fashion by image analysis.

Proliferation index has been found to be of prognostic value in a variety of soft tissue tumors [7–9]. Oshiro et al. [2] stated that proliferating cell nuclear antigen (PCNA) score could not by itself distinguish benign from malignant fibrous lesions, but they believed it to be an independent predictor for separating abdominal and extra-abdominal fibromatoses. They studied a total of 20 fibromatoses and found PCNA values to be higher in extra-abdominal than abdominal fibromatoses. Extra-abdominal fibromatoses are known to have a higher recurrence rate than abdominal fibromatoses. Our study contained an insufficient number of abdominal fibromatoses for statistically significant analysis, but no relationship was detected between proliferation index as determined by MIB-1 and recurrence (Table I). No level of MIB-1 reactivity could be found which stratified fibromatoses into groups at high or low risk for recurrence.

ER level has been correlated with prognosis for carcinoma of the breast [10,11]. ER levels above 10 fmol/mg have been found to stratify breast cancer patients into favorable and unfavorable prognostic categories [4]. ER has been detected in the cells of some fibromatoses, and clinical data would indicate that some of these lesions are hormonally modulated [1]. We found no relationship between ER level and likelihood of recurrence in our series of 52 cases when a cutpoint of either 10 or 30 fmol/mg was used (Table II).

Recent studies have detected mutations in the p53 gene in some soft tissue sarcomas [12,13] and fibromatoses [2]. These mutations result in accumulation of p53 within the cell nuclei which is detectable by immunohistochemistry. Malignant behavior has been correlated with p53 protein accumulation in some soft tissue sarcomas [2,14], but no link with incidence of recurrence could be made. We found increased levels of p53 protein to be associated with recurrence in our study of 52 cases of fibromatosis ($P = 0.06$). This association appeared to be independent of location of the lesion. It has been suggested that p53 overexpression in benign lesions is due to accumulation of wild-type p53 and differs from the cause of increased stainable p53 protein in malignancies [2]. We were unable to define the nature of the accumulation seen in the fibromatoses of our study.

The Rb gene codes for a nuclear phosphoprotein involved in cell cycle control [15]. Studies of Rb protein status have shown a relationship between the absence of Rb protein and poor prognosis in some neoplasms [16–21]. A relationship has also been demonstrated between absence of Rb protein and resistance to some chemotherapy agents [22]. While Rb protein was immunohistochemically absent in 13 of 34 (38%) fibromatoses, we were unable to find any correlations between recurrence and Rb protein expression ($P = 0.49$).

The cellular proto-oncogene, C-*src*, was isolated through studies of the Rous chicken fibrosarcoma. C-*src* encodes a protein, p60c-*src*, which can phosphorylate proteins with tyrosine residues [23]. The normal role of p60c-*src* remains unclear, but it appears to play a role in the cell cycle and proliferation in a number of normal and neoplastic tissues [23,24]. Using immunohistochemical methods, we were unable to detect p60c-*src* in the 20 fibromatoses we studied for this protein. The significance of this finding is unclear from our data.

Aggressive fibromatoses are associated with several cytogenetic abnormalities including loss of the Y chromosome, abnormalities of chromosome 5, and other complex clonal chromosomal abnormalities [25]. While such changes support a neoplastic nature for fibromato-

ses, little data are available correlating behavior and cytogenetic abnormalities in fibromatoses.

In our series of 52 cases of fibromatosis, only lesion depth and p53 level correlated with recurrence. These two factors appeared to be independent variables. The majority of the variables tested in this series were related to proliferation and the cell cycle. Proliferation index (MIB-1) directly measures the percentage of cells undergoing the replication phases of the cycle cell while p53 and Rb gene products are involved in control of replication. While p53 level was correlated with recurrence, neither Rb protein level nor proliferation index was predictive of recurrence.

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